INSTRUCTIONS

Pierce™ Agarose ChIP Kit

26156

2216.7

Number	Description
26156	**Pierce Agarose ChIP Kit,** contains sufficient reagents to perform ChIP reactions for 30 target proteins from 10 sample conditions

Kit Contents:

ChIP Grade Protein A/G Plus Agarose, 0.65mL; store at 4°C

IP Dilution/Wash Buffer (5X), 13mL, store at 4°C

IP Wash Buffer 3 (5X), 4.5mL; store at 4°C

IP Elution Buffer (2X), 4.5mL; store at 4°C

Sodium Chloride (5M), 3mL; store at 4°C

Column Accessory Pack, 3 packs, 10 spin columns, 20 collection tubes and 10 plugs; store at room temperature or 4°C

Microcentrifuge Tubes, 1.5mL, 75 tubes; store at room temperature or 4°C

DNA Clean-Up Column and Reagents, 40 purifications; store at room temperature or 4°C

- **DNA Clean-Up Column,** 40 columns
- **DNA Column Binding Solution,** 30mL
- **DNA Column Wash Solution,** 6mL
- **pH Indicator,** 0.8mL
- **DNA Column Elution Solution,** 5mL

Anti-RNA Polymerase II Antibody, 25µL; store at -20°C

Normal Rabbit IgG, (1mg/mL), 10µL; store at -20°C

ChIP Positive Control Primers (GAPDH promoter), 100µL; store at -20°C

Pierce Chromatin Prep Module (Product No. 26158)

Membrane Extraction Buffer, 10mL; store at 4°C

Nuclear Extraction Buffer, 10mL; store at 4°C

MNase Digestion Buffer, 5mL; store at 4°C

MNase Stop Solution, 0.5mL; store at 4°C

Halt™ Protease and Phosphatase Inhibitor Cocktail EDTA-free (100X), 4 × 90µL microtubes; store at 4°C

Glycine Solution (10X), 15mL; store at room temperature

PBS (20X), 15mL; store at 4°C

Micrococcal Nuclease (ChIP Grade) (10 U/µL), 25µL; store at -20°C

Proteinase K (20mg/mL), 0.25mL; store at -20°C

DTT (7.7mg), Lyophilized, 1 vial; store at room temperature or 4°C

Storage: Upon receipt store contents of yellow pouch at -20°C. Store all other components as indicated. The kit is shipped at ambient temperature.

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Introduction

The Thermo Scientific Pierce Agarose ChIP Kit provides a simple, fast and reproducible method to perform chromatin immunoprecipitation (ChIP) assays. ChIP assays identify links between the genome and the proteome by monitoring transcription regulation through histone modification[1] (epigenetics) or transcription factor-DNA binding interactions. The strength of the ChIP assay technique is its ability to capture a snapshot of specific protein-DNA interactions as they occur in living cells and then quantitate the interactions using standard or quantitative PCR.[2,3]

The kit contains all reagents to perform a successful ChIP assay. The specially titrated and tested micrococcal nuclease digests the DNA, eliminating variable results caused by the traditional method of sonication. The advantages of enzymatic digestion include reproducibility of digestion, control of the reaction, and easy titration of the enzyme for each specific cell type. The specially blocked ChIP Grade Pierce Protein A/G Plus Agarose Resin provides high-binding capacity and low background. Performing the ChIP, wash, and elution steps in the included spin columns is fast, convenient and minimizes sample loss. Using these columns and optimized reagents, the Pierce ChIP Kit protocol is streamlined to achieve complete crosslink reversal, protein digestion, and DNA purification with minimal time and sample handling.

Additional Materials Required

- Mammalian cell culture reagents and equipment
- Chemical fume hood
- ChIP-qualified primary antibody against the DNA binding protein of interest
- Cell scrapers
- Thermomixer or heat block
- 50 and 15mL conical tubes
- 16% Formaldehyde (Product No. 28906)
- DMSO (optional)
- Nuclease-free water
- Ethanol (95-100%)
- Standard or quantitative PCR reagents and equipment
- Oligonucleotide primers specific to the gene promoter of interest

Protocol for ChIP Assay

Note: Read the entire protocol before beginning the assay.

A. Experimental Design

- When designing a ChIP experiment, consider the number of immunoprecipitations desired from the chromatin sample and the number of cell culture conditions (e.g., drug-treated vs. non-treated). For accurate comparison, immunoprecipitate the same target proteins and controls from each cell culture condition.
- Performing a ChIP using the Normal Rabbit IgG is an effective negative control.
- The included anti-RNA polymerase II antibody and GAPDH primers provide a positive control for assay technique and reagent integrity. Perform a single RNA polymerase II immunoprecipitation on isolated chromatin from the control (non-treated) cell culture condition. Amplify the resulting enriched DNA with the provided GAPDH primer pair. The strength of the RNA Polymerase II IP signal should be > 50% relative to the total input control in a well-functioning assay (see Troubleshooting Section).
- If performing multiple ChIPs from a single cell culture condition, crosslinked chromatin may be prepared in bulk. Scale the reagent amounts according to the number of ChIPs desired (Table 1) and sub-divide the chromatin sample after diluting with the 1X IP Dilution Buffer. The total input control obtained from the cell culture condition is common to this set of ChIPs.

Table 1. Reagent volumes to use based on the number of ChIPs.

Example number of HeLa cells	2×10^6	6×10^6	2×10^7	6×10^7
Number of ChIPs	1	3	10	30
1X PBS	2.1mL	6.3mL	21mL	63mL
Lysis Buffer 1	0.1mL	0.3mL	1mL	3mL
Lysis Buffer 2	0.05mL	0.15mL	0.5mL	1.5mL
MNase Digestion Buffer Working Solution	0.1mL	0.3mL	1mL	3mL
1X IP Dilution Buffer	0.45mL	1.2mL	4.5mL	12mL
IP Wash Buffer 1	0.5mL	1.5mL	5mL	15mL
IP Wash Buffer 2	1mL	3mL	10mL	30mL
IP Wash Buffer 3	0.5mL	1.5mL	5mL	15mL
1X IP Elution Buffer	0.15mL	0.45mL	1.5mL	4.5mL

B. Material Preparation

- Prepare the reagents listed below once and use for all 30 ChIP reactions.

Glycine Solution (10X)	If precipitate formed, warm solution to 37°C in a water bath for 30 minutes and vortex before use.
PBS	Dilute PBS (20X) to 1X with nuclease-free water.
1M DTT	Add 50µL of ultrapure water to the DTT. Store this solution at -20°C for up to 6 months.
DNA Column Binding Buffer	Add 120µL of pH Indicator Solution to the vial of 30mL DNA Column Binding Solution and mix well. Store at room temperature.
DNA Column Wash Buffer	Add 24mL of ethanol (95-100%) to the vial of 6mL DNA Column Wash Solution provided and mix well. Store at room temperature.

- The amounts listed below are for preparing one ChIP. If you are preparing chromatin in bulk or performing multiple ChIPs, multiply the reagent amounts by the number of ChIPs being performed (see Table 1).

Lysis Buffer 1	Add 1µL of the Halt Cocktail to 100µL of Membrane Extraction Buffer in a microcentrifuge tube and place on ice.
Lysis Buffer 2	Add 0.5µL of the Halt Cocktail to 50µL of Nuclear Extraction Buffer in a microcentrifuge tube and place on ice.
MNase Digestion Buffer Working Solution	Add 0.1µL of 1M DTT to 100µL of MNase Digestion Buffer and place tube at room temperature.
1X IP Dilution Buffer	Add 100µL of IP Dilution/Wash Buffer (5X) and 5µL of Halt Cocktail to 395µL of nuclease-free water. Store at 4°C.
IP Wash Buffer 1	Prepare 0.5mL by combining 0.1mL IP Dilution Buffer/Wash Buffer (5X) with 0.4mL nuclease-free water. Store at 4°C.
IP Wash Buffer 2	Prepare 1mL by combining 0.2mL IP Dilution/Wash Buffer (5X) and 70µL Sodium Chloride (5M) with 0.73mL of nuclease-free water. Store at 4°C.
1X IP Wash Buffer 3	Prepare 0.5mL by combining 0.1mL of IP Wash Buffer 3 (5X) with 0.4mL of nuclease-free water. Store at 4°C.
1X IP Elution Buffer	Warm the IP Elution Buffer (2X) in a 37°C water bath until fully dissolved. For each IP and total input control, prepare 150µL of 1X IP Elution Buffer by combining 75µL of IP Elution Buffer (2X) with 75µL nuclease-free water. Store at room temperature.

C. Crosslinking and Cell Pellet Isolation

Note 1: Perform all centrifugations at room temperature.

Note 2: This procedure is for one ChIP. Multiply reagent amounts by the number of ChIPs being performed (see Table 1).

1. Culture adherent mammalian cells and treat as desired.

   Optional: If you are unfamiliar with cell type being used, culture an extra dish of cells for determining cell number. Before crosslinking, trypsinize and determine the cell number from the extra dish of cells.

2. To each dish containing cell culture media, add sufficient quantity of 16% formaldehyde to obtain a final concentration of 1% formaldehyde.

   Caution: Formaldehyde is a skin irritant and the fumes are toxic. Use proper personal protective, laboratory safety and disposal equipment.

3. Mix well by gently swirling the dish. Incubate at room temperature for 10 minutes in a chemical fume hood.
4. To each dish containing cell culture media and formaldehyde, add Glycine Solution (10X) to a final concentration of 1X. Mix well by gently swirling the dish. Incubate at room temperature for 5 minutes in the chemical fume hood.
5. Aspirate formaldehyde/glycine-containing media in the fume hood. Properly dispose the formaldehyde-containing waste.
6. Wash the cells twice with one media volume of ice-cold PBS, removing each wash by aspiration.
7. Add 10µL of the Halt Cocktail to 1mL of ice-cold PBS. Add the solution to the cells, and detach cells by scraping. Transfer the cell suspension to a 1.5mL microcentrifuge tube using a pipette
8. Centrifuge tubes at 3000 × *g* for 5 minutes.
9. Remove the PBS. Store the cell pellet(s) at -80°C, or proceed directly to Section D: Lysis and MNase Digestion.

D. Lysis and MNase Digestion

Note: For best results, empirically determine the optimal crosslinking time and Micrococcal Nuclease digestion conditions for each cell type (see Appendix A).

1. Use the crosslinked cells prepared above. If frozen, thaw cells on ice.
2. Add100µL Lysis Buffer 1 containing protease inhibitors to the cell pellet and pipette up and down to break up the pellet. Vortex the tube for 15 seconds and incubate on ice for 10 minutes.
3. Centrifuge at 9000 × *g* for 3 minutes and remove the supernatant.
4. Resuspend nuclei in 100µL of MNase Digestion Buffer Working Solution.
5. Add 0.25µL of Micrococcal Nuclease (ChIP Grade) (10 U/µL), vortex the tube and incubate in a 37°C water bath for 15 minutes, mixing by inversion every 5 minutes.
6. Add 10µL of MNase Stop Solution to stop the reaction, vortex briefly and incubate on ice for 5 minutes.
7. Centrifuge at 9000 × *g* for 5 minutes to recover the nuclei. Remove the supernatant.
8. Resuspend nuclei in 50µL of Lysis Buffer 2 containing protease/phosphatase inhibitors and incubate on ice for 15 minutes, vortexing for 15 seconds every 5 minutes.
9. Centrifuge at 9000 × *g* for 5 minutes and transfer the supernatant, containing the digested chromatin, to a new 1.5mL tube. Proceed to the immunoprecipitation or store samples at -80°C.

E. Immunoprecipitation

1. Transfer 5µL of the supernatant containing the digested chromatin to a 1.5mL tube and store at -20°C. This is the 10% total input sample from one ChIP.

   Note: If you are preparing chromatin in bulk, this sample can be used as a common input control for all IPs from the bulk preparation.

2. Transfer the remaining 45µL of supernatant to 450µL of 1X IP Dilution Buffer.

   Note: If you are preparing chromatin in bulk, unused supernatant may be stored at -80°C for later use.

3. For each IP, add 500µL diluted lysate to a plugged spin column and add primary antibody. The following amounts of antibody are recommended when using 2 ×10^6 HeLa cells/IP.

   Positive control IP (included): add 10µL Anti-RNA Polymerase II Antibody

   Negative control IP (included): add 1 to 2µL of Normal Rabbit IgG

   Target-specific IP(s): The typical concentration is 1-10µg antibody for each IP; however, titration of antibody concentration to obtain the best signal-to-noise ratio is required.

4. Incubate IP reactions for 2 hours to overnight at 4°C on a rocking platform.

   Note: For low-abundant proteins, an overnight incubation greatly increases signal.

5. Cap and vortex the tube of ChIP Grade Protein A/G Plus Agarose to obtain a uniform suspension. Using a wide-bore or cut pipette tip, add 20µL of the agarose resin to each IP and incubate for 1 hour at 4°C on a rocking platform.

6. After resin incubation, remove and set aside the column plug. Place the column into a 2mL collection tube.

7. Centrifuge tube at 3000 × *g* for 30 seconds. Remove column from the collection tube, discard the column flow-through and set aside the collection tube.

8. Re-insert the same column plug, apply 0.5mL of IP Wash Buffer 1, cap the column and incubate at 4°C for 5 minutes on a rocking platform.

9. Remove the column plug and set aside. Place the column into the same collection tube.

10. Centrifuge at 3000 × *g* for 30 seconds. Remove column from the collection tube, discard the column flow-through and set aside the collection tube.

11. Repeat steps 8-10 twice with IP Wash Buffer 2.

12. Repeat steps 8-10 once with IP Wash Buffer 3.

13. To remove residual Wash Buffer, insert column into the collection tube and centrifuge at 3000 × *g* for 1 minute.

F. IP Elution

1. Re-fit column plug, add 150µL 1X IP Elution Buffer to the washed resin, cap the column, and incubate at 65°C for 30 minutes with shaking. If a thermomixer is unavailable, incubate the column in a heat block set a 65°C for 40 minutes. Re-suspend the beads by flicking the tube every 10 minutes.

2. During the elution step, prepare a 1.5mL microcentrifuge tube for each IP containing 6µL of 5M NaCl and 2µL of 20mg/mL Proteinase K.

3. Thaw 10% total input sample(s) and add 150µL of IP Elution buffer, 6µL of 5M NaCl and 2µL of 20mg/mL Proteinase K. Place tubes at room temperature until the next step.

4. Following the 65°C incubation, remove the column from the heat block, open the cap, remove the plug, and place the column in the previously prepared 1.5mL collection tube containing the NaCl and Proteinase K, close the column cap and centrifuge at 6000 × *g* for 1 minute.

   Note: After incubation, it is essential to open the column cap before plug removal to equalize the pressure within the column. Failure to open the cap before plug removal will result in sample loss.

5. Discard the columns. Cap the 1.5mL centrifuge tubes, vortex all IP and total-input samples and place in a 65°C heat block for 1.5 hours.

G. DNA Recovery

1. To each eluted IP and total input sample, add 750µL of DNA Binding Buffer.

2. Pipette 500µL of each sample into a DNA Clean-Up Column inserted into a 2mL collection tube. Centrifuge the columns at 10,000 × *g* for 1 minute and discard the flow-through.

3. Pipette the remaining sample into the same DNA Clean-Up Column. Centrifuge the columns at 10,000 × *g* for 1 minute and discard the flow-through.

4. Place the column back into the collection tube and add 750µL of DNA Column Wash Buffer. Centrifuge the columns at 10,000 × *g* for 1 minute and discard the flow-through.

5. Place the column back in the empty collection tube and centrifuge the columns at 10,000 × *g* for 2 minutes.
6. Place the column in a new 1.5mL centrifuge tube and pipette 50µL of DNA Column Elution Solution directly into the center of each column.
7. Centrifuge the column at 10,000 × *g* for 1 minute and discard the column. The resulting solution is the purified DNA. Proceed to PCR or QPCR detection (see Appendix B).

Troubleshooting

Problem	Possible Cause	Solution
Chromatin fragments are too large (> 1000 base pairs)	Crosslinking time was too long	Shorten crosslinking time
	Cell to Micrococcal Nuclease (MNase) ratio was too high	Increase amount of MNase or decrease cell number (refer to the MNase digestion optimization protocol in Appendix A)
Chromatin fragments are too small (< 200 base pairs)	Cell to MNase ratio was too low	Decrease amount of MNase or increase cell number (refer to the MNase digestion optimization protocol in Appendix A)
Chromatin not recovered from nucleus	Cell type requires more stringent handing to better lyse and release chromatin from nucleus	Increase incubation with nuclei lysis buffer to 30 minutes and vortex for 30 seconds every 5 minutes
		Following the nuclear lysis step, sonicate the sample for 60 seconds using a 1/8 probe. Perform on wet ice in 3 pulses of 20 seconds with 30 second pauses between
		Following the nuclear lysis step, dounce the sample 20 times in a glass dounce homogenizer
No or low PCR signal in the total input control samples	PCR amplification conditions were not fully optimized	Optimize PCR conditions using samples known to contain the target amplicon
		Check primer design
	Insufficient amount of sample DNA added to the PCR reaction	Increase the amount of sample DNA added to the PCR reaction
No or low PCR signal in the positive control IP samples	Insufficient chromatin amount in the IP reaction	Use at least 25µg of chromatin for each IP
	Insufficient antibody incubation time	Incubate antibody overnight
	Incomplete elution from the Protein A/G agarose resin	Perform elution at 65°C and increase frequency of mixing
PCR signal of the positive and negative control IP samples are equivalent	Insufficient washing of the IP complex	Include an additional wash with Buffers 2 and 3
	Excess chromatin or antibody added to the IP	Add less chromatin or antibody
	PCR amplification was measured outside the linear range of amplification	Decrease the number of amplification cycles used in the PCR reaction

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No or low PCR signal in the experimental IP samples	Insufficient amount of sample DNA added to the PCR reaction	Add more sample DNA to the PCR reaction
	Insufficient amount of antibody added to the IP	Add more antibody to the IP
	Antibody does not function in an IP	Verify that the antibody is qualified for ChIP or IP applications

Note: Increasing the stringency of the nuclear lysis is not recommended unless there is no signal or low signal-to-noise in the IP. Excess sample can result in high background, decreasing the signal of the specific signal.

Appendix A: Optimization of micrococcal nuclease digestion

1. Prepare crosslinked cell pellet from 10^7 cells as described in Section C.
2. Follow the lysis procedure in Section D, Steps 1-4; however, use 0.5mL of the Lysis Buffer 1, Membrane Extraction Buffer and MNase Digestion Buffer Working Solution.
3. Transfer 100µL of the resuspended nuclei into five tubes (2×10^6 nuclei/tube), labeling the tubes 0, 1, 2, 4 and 6.
4. Add 2µL of the Micrococcal Nuclease (ChIP Grade) to 18µL of MNase Digestion Buffer Working Solution (1:10 dilution).
5. Add 0, 1, 2, 4 and 6µL of the diluted Micrococcal Nuclease to the corresponding tubes of nuclei and incubate for 15 minutes in a 37°C water bath mixing by inversion every 5 minutes.
6. Add 20µL MNase Stop Solution to each tube and incubate on ice for 5 minutes.
7. Centrifuge at 9000 × *g* for 5 minutes to recover the nuclei and remove the supernatant.
8. Resuspend nuclei in 50µL of Nuclear Extraction Buffer and incubate on ice for 15 minutes, vortexing for 15 seconds every 5 minutes.
9. Centrifuge at 9000 × *g* for 5 minutes.
10. Prepare five 1.5mL microcentrifuge tubes labeled 0, 2, 4 and 6 each containing 6.6µL Nuclease Free Water, 2.4µL of 5M NaCl, and 1µL of Proteinase K (20mg/mL).
11. Transfer 50µL of each nuclear supernatant, containing digested chromatin, to the corresponding tube.
12. Vortex each tube for 10 seconds and incubate the tubes at 65°C in a heat block for 1.5 hours.
13. Analyze 15-25µL of each sample and DNA size marker by agarose gel electrophoresis. Optimal digestion should yield fragments from 200 to 1000 base pairs with a more intense ladder of bands occurring at approximately 160, 320, and 480 base pairs, which corresponds to the 1, 2 and 3 nucleosome units.

Appendix B: Real-time PCR analysis using positive control primers

Recommendations:

- Use filter tips, gloves, and nuclease-free reagents to prevent contamination.
- Using hot-start Taq polymerase and validated PCR master mix greatly reduces PCR artifacts and increases reaction efficiency.
- The Positive Control Primers included are designed to amplify a region of the human GAPDH promoter close to the transcription start site. The primers are validated for detecting RNA polymerase II binding to the GAPDH promoter in human-derived cells and tissue. Detection of other Protein:DNA interactions using these primers is not validated.
- When designing primers, using reputable primer design software greatly increases successful PCR detection.
- Follow the manufacturer's recommendations when programming the thermocycler and collecting real-time data.
- Use a standard curve of serially diluted genomic DNA to evaluate PCR efficiency and linear amplification.
- Amplify each sample in triplicate to control for pipetting error.

Recommended reaction conditions for quantitative real-time PCR (qPCR) with the GAPDH control:

Reagent	Volume/reaction (µL)
Thermo Scientific 2X Absolute QPCR SYBR Green Fluorescein Mix	12.5
ChIP Positive Control Primers (GAPDH Promoter)	1.0
Template DNA	5.0
Nuclease-free Water	6.5

Thermocycler Amplification Settings:

Step 1: 95°C for 15 minutes.

Step 2: 95°C for 15 seconds.

Step 3: 62°C for 1 minute (collect real-time data).

Step 4: Repeat Steps 2 to 3 for 40 cycles.

Related Thermo Scientific Products

See the website for a listing of ChIP-validated antibodies.

28908 **16% Formaldehyde (w/v), Methanol-free,** 10 × 1mL

20688 **Dimethylsulfoxide (DMSO), Sequanal grade,** 950mL

26158 **Pierce Chromatin Prep Module**

26159 **ChIP Grade Protein A/G Plus,** 0.65mL

26160 **Proteinase K Solution (20mg/mL),** 0.25mL

28372 **BupH™ Phosphate Buffered Saline Pack,** 40 packs

78425 **Halt Protease Inhibitor Cocktail, EDTA-Free (100X),** 24 vials

78443 **Halt Protease and Phosphatase Inhibitor Cocktail, EDTA-Free (100X),** 24 vials

78833 **NE-PER Nuclear and Cytoplasmic Extraction Kit**

20148 **LightShift™ Chemiluminescent EMSA Kit**

23225 **Pierce BCA Protein Assay Kit**

22662 **Pierce 660nm Protein Assay Kit**

AB1219 **Absolute™ QPCR SYBR™ Green Fluorescein Mix**

AB-0900/w **Thermo-Fast™ 96 Semi-Skirted PCR Plate, opaque white**

AB-1170 **ABsolute QPCR Seal**

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General References

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